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A New Variety of the Genus *Panax* from Southern Yunnan, China and Its Nucleotide Sequences of 18S Ribosomal RNA Gene and *mat*K Gene

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A new variety of *Panax* L., *P. vietnamensis* Ha & Grushv. var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai is described, together with its nucleotide sequences of 18S rRNA gene and *mat*K gene. On the basis of both gene sequences, the phylogenetic relationship among the new variety and other 6 *Panax* taxa has been investigated. This new variety exhibits a close relationship with *P. vietnamensis* in morphological characters as well as molecular evidences, but differs from it in having flat, fuscous or vaccinous disk and completely separated styles in 2-styled flowers, besides the unique nucleotide sequences of *mat*K gene. Its somatic chromosome number is 2n = 2x = 24.

Key words: chromosome number, 18S rRNA gene, *mat*K gene, new variety, *Panax vietnamensis* var. *fuscidiscus*, phylogenetic relationship.

Introduction

The taxonomic treatment of the genus *Panax* L. has long been considered controversial because of morphological variation within taxa and the high frequency of morphologically intermediate plants (Hara 1970, Hoo and Tseng 1973, 1978, Zhou et al. 1975). To date, the most significant taxonomic treatment of the genus *Panax* has been proposed by Wu in Zhou et al. (1975), in which six independent species and four varieties of *P. japonicus* were determined according to the morphological characters, chemical constituents and geographic distribution. In the course of our systematic studies on the genus *Panax*, a collaborative botanical expedition was conducted in Yunnan province, China from July to August 1999. During the investigation, we collected several specimens of a *Panax* plant in Jinping county, in the southern part of Yunnan province. At first glance, this plant was very similar to *P. japonicus* C.A.Mey. as both of them possess a bamboo-like rhizome. Of the four already described varieties of *P. japonicus*, only *P. japonicus* var. *japonicus* and *P. japonicus* var. *angustifolius* (Burk.) Cheng & Chu have been recorded for the Jinping county region (Zhou et al. 1975, Wu et al. 1979). Gene sequence analysis discussed in this paper showed, however, that the sequences of the nuclear 18S ribosomal RNA gene and chloroplast *mat*K gene of these specimens were considerably different from those of *P. japonicus*, but were close to those of *P. vietnamensis* Ha & Grushv.

Consequently, we critically examined the morphology of the specimens collected from Jinping county and several cultivated specimens sprouted from rhizomes of a crude drug sample purchased in the region. It was found that they were markedly different from *P. japonicus*, but similar to *P. vietnamensis* in characters of leaflet, inflorescence and flower. However, the Jinping specimens differed from *P. vietnamensis* in having a flat, fuscous or vaccinous disk (joint surface between ovary and style) and completely separated styles in the 2-styled flowers, as well as the unique nucleotide sequences for the *mat*K gene. Accordingly, a new variety under *P. vietnamensis* has been advocated and named as *P. vietnamensis* Ha & Grushv. var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q. Cai.

In the present paper, this new variety is described on the basis of morphology, together with nucleotide sequences of the 18S rRNA and *mat*K genes and the somatic chromosome number. At the same time, the phylogenetic relationship between the new variety and six other *Panax* taxa originally presented in Komatsu et al. (2001) is discussed after the phylogenetic tree based on *mat*K gene sequences was reconstructed to include the new taxon. All specimens examined here excepting *P. pseudo-ginseng* Wall. subsp. *himalaicus* H.Hara are identified following Wu's classification in Zhou et al. (1975), which has been widely applied in China.

Materials and Methods

Materials

Six samples of *P. vietnamensis* var. *fuscidiscus* were examined, including four plant specimens collected from the type locality in Jinping county (K. Komatsu et al. Y276–1, Y276–2, Y276–3, Y276–4), and two individuals which were one rhizome and one root as the composition of a crude drug sample named "Ye-Sanchi" obtained from Sankeshu, a village market 100 km far from Jinping (TMPW No. 19759). The voucher specimens were stored in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University (TMPW) and the Herbarium, School of Pharmaceutical Sciences, Peking University (PEM).

Isolation of total DNA, PCR amplification and sequencing reaction

Total DNA was extracted from dried leaves of the plant specimens using the DNeasy™ plant Mini Kit (QIAGEN, Germany), and from the crude drug samples by the method described in a previous paper (Fushimi et al. 1997). The amplification of complete 18S rRNA gene and *mat* K gene regions via the polymerase chain reaction (PCR), the purification of PCR products and

Table 1. Sequencing primers of 18S rRNA gene and *mat*K gene of *Panax* species

Primer of 18S rRNA gene	Primer sequence (5' to 3')	Primer of *mat*K gene	Primer sequence (5' to 3')
Forward primers		Forward primers	
IRD-18S 5'F	GAT CCT GCC AGT AGT CAT ATG C	IRD-*mat*K 34F	GCA CTT GCT CAT GAT CAT GG
IRD-FZ840	GTT GGC CTT CGG GAT CGG AG	IRD-*mat*K 748F	AAG GAT CCT TTC ATG CAT TAT
IRD-FZ1308	TTT GTC TGG TTA ATT CCG TT		
Reverse primers		Reverse primers	
IRD-RZR460	TTA TTG TCA CTA CCT CCC CG	IRD-*mat*K 514R	TAT TCC AAT TAC AAT ACT CGT
IRD-RZR1028	CCT GGT CGG CAT CGT TTA TG	IRD-*mat*K 1219R	GTC GAA GTA TAT ACT TTA TTC G
IRD-18S 3'R	GAC CTA CGG AAA CCT TGT TAC G		

sequencing reaction using each set of fluorescent-labeled sequencing primers (Table 1) for 18S rRNA and *mat*K genes, respectively, were performed as described in a previous paper (Komatsu et al. 2001).

Sequencing and phylogenetic analysis

Each sequence was determined directly and analyzed using a 4000L DNA sequencer (LI-COR, U.S.A.). The nucleotide sequence data of the 18S rRNA and the *mat*K genes were recorded in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers: AB080243 for 18S rRNA gene and AB080244 for *mat*K gene.

The phylogenetic trees were reconstructed using the computer program PAUP* (Version 4.0b6a, Sinauer Assoc. Inc., U.S.A.). Parsimony analysis was performed using the Heuristic search method, with tree-bisection-reconnection (TBR) branch-swapping, MULTREES, a random addition sequence of 500 replicates, and with *Acanthopanax senticosus* (Accession numbers in GenBank: AB080245 for 18S rRNA gene, AB080246 for *mat*K gene) as outgroup. Bootstrap (1000 replications) analysis was performed to estimate the confidence of topology of the consensus tree.

Morphological observation

The morphological characters of plant specimens, crude drug sample (TMPW No. 19759, underground parts only), and several cultivated specimens sprouted from the rhizomes of the crude drug sample were observed in detail with the aid of loupe and stereomicroscope.

Observation of somatic chromosome numbers

The method for observation of chromosome numbers was modified from that of Liu and Ohba (2000). Root tips were pretreated with 0.002M 8-hydroxyquinoline for 3 hr, then were fixed into 1:3 fresh mixture of 45 % acetic acid and 99.5 % ethanol for 40 min at 4 °C and subsequently soaked in 99.5 % ethanol at −20 °C for restoring. Before observation, root-tips were macerated in 5 N HCl for 5 min, and then stained with 1 % aceto-orcein for 1 hr at room temperature.

Results

Gene sequence analysis

All plant specimens and individuals from the crude drug sample of *P. vietnamensis* var. *fuscidiscus* (PVF) possessed completely identical sequences for the nuclear 18S rRNA gene and for the chloroplast *mat*K gene, respectively, demonstrating intra-taxon stability of PVF on these two genes.

The 18S rRNA gene sequence of PVF was found to be 1809 base pairs in length – the same as those of other six *Panax* taxa reported (Komatsu et al. 2001), and showed an identical sequence to that of *P. vietnamensis* (PV) and *P. quinquefolius* L. (PQ). Therefore, it was different from the sequence of *P. ginseng* C.A.Mey. (PG) at three nucleotide sites (the nucleotide positions 497, 499 and 501 from upstream), from *P. japonicus* (PJJ) at two nucleotide sites (499, 712), and from *P. japonicus* var. *major* C.Y.Wu & K.M. Feng (PJM) and *P. pseudo-ginseng* subsp. *himalaicus* (PPH) at one nucleotide site (499).

The *mat*K gene sequence of PVF was found to be 1512 base pairs long (including the terminal codon), the same length as in the other six *Panax* taxa (Komatsu et al. 2001), and showed 2–8 sites of nucleotide difference from those six taxa, most of which were clustered at the 5' end of the gene (Fig. 1). PVF revealed the same sequences as PV, PJM and PPH at nucleotide positions 357, 396, 606, 1205 and 1416, differing from those common to PG, PJJ and PQ. PVF and PV exhibited the same nucleotides at positions 356 and 1491, but differed from each other at positions 104 and 1043 (Fig. 1). The

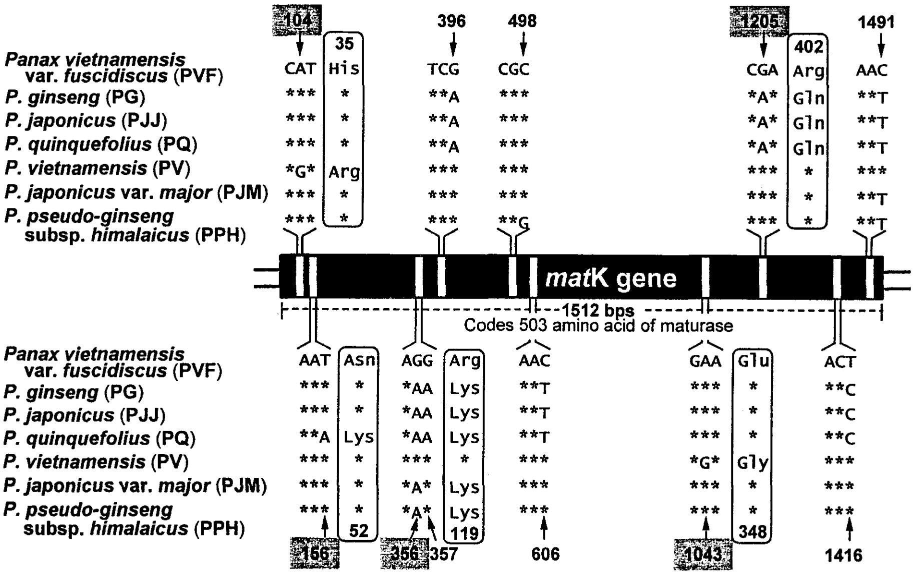

Fig. 1. Comparison of *mat*K gene sequences among *Panax vietnamensis* var. *fuscidiscus* (PVF) and six *Panax* taxa. Base substitutions present at nucleotide positions 104, 156, 356, 357, 396, 498, 606, 1043, 1205, 1416 and 1491 from upstream. The shaded positions are nonsynonymous substitutions which induce amino acid alteration in translated protein (maturase), and corresponding alteration of amino acid residues are shown in the next frame. Excepting PVF, the sequences of the other six taxa have been reported in our previous paper (Komatsu 2001). The localities of these samples are as follow: *P. ginseng*: Japan; *P. japonicus*: Japan; *P. quinquefolius*: Japan (originated from Canada); *P. vietnamensis*: Vietnam; *P. japonicus* var. *major*: Yunnan, China; *P. pseudo-ginseng* subsp. *himalaicus*: Arunachar Pradesh, India.

total 11 nucleotide substitution sites among all seven *Panax* taxa included five sites of nonsynonymous substitutions (104, 156, 356, 1043 and 1205), and six sites of synonymous substitutions (silent substitutions: 357, 396, 498, 606, 1416 and 1491) (Fig. 1). The high nonsynonymous substitution is due to the high substitution frequency at the first and second codon positions of *mat*K gene (Hilu and Liang 1997).

Based on the *mat*K gene sequence, phylogenetic relationship among the new variety, PVF, and the other six taxa was reconstructed by the maximum parsimony method (Fig. 2). The phylogenetic trees showed that the seven *Panax* taxa were divided into two clusters. PVF and PV formed a sister group supported by a high bootstrap value (87 %), and belonging to the different cluster from PG, PJJ and PQ.

Morphological characteristics and chromosome number

The result of morphological observation on the new plant is given in the description of the new variety.

This variety resembles *P. vietnamensis* in general characters, but the disk is flat, fuscous or vaccinous in color and the styles are completely separated in 2-styled flowers. The fuscous or vaccinous disk found in *Panax vietnamensis* var. *fuscidiscus* is a unique characteristic in the genus *Panax*.

The new variety was found to be a diploid taxon with a chromosome number of $2n=2x=24$.

Discussion

This new variety possesses a bamboo-like horizontally creeping rhizome, which was customarily considered as a representative character of PJJ. Moreover, the leaflet of PVF was ovate-oblong or elliptic in shape with a long caudate-acuminate apex, showing superficial similarity to those of PJJ, which are ovate or elliptic with a cuspidate apex. This may be the reason that this new variety, *P. vietnamensis* var. *fuscidiscus* had not been recognized properly in previous taxonomic treatments. PVF is characterized in having only a single umbelliform inflorescence in which 70–100 little flowers are fascicled, the flowers were found to have 1 or 2 separated styles; a unilocular or bilocular ovary; the disk was flat and fuscous or vaccinous in color; and conspicuous bristles were present along the veins of both surfaces of the leaflets. These characters differed considerably from PJJ which possessed several inflorescences consisting of 20–50 little flowers each with, 2–4 separate styles and a 2–4 locular ovary, short and sparse bristles along the veins of both surfaces. The morphology of PVF was very similar to *P. vietnamensis* (PV) – recently discovered in central Vietnam (Ha and Grushvitzky 1985), which possessed of more than 50 flowers (50–120) in an inflorescence, only 1 or 2 styles, a unilocular or bilocular ovary, as well as the caudate-acuminate leaflet apex. However, the subconcave and greenish-white disk, the 2-styles united at the basal part, and rarely occurring several inflorescences of PV differed clearly from those of PVF. The combinations of characters in these three taxa suggested that PVF was more similar to PV than PJJ, therefore, we advocated it as a variety of PV. The fuscous or vaccinous disk is the distinctive feature of PVF since this character has not been observed in any other *Panax* plants. In addition, PVF was also shown to be distinctive in the results of our gene sequence analysis. Although its 18S rRNA gene se-

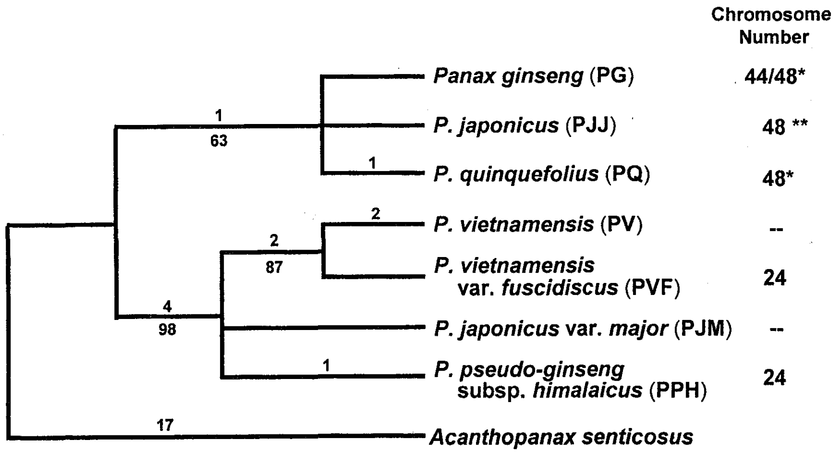
Chromosome Number
Panax ginseng (PG) 44/48*
P. japonicus (PJJ) 48 **
P. quinquefolius (PQ) 48*
P. vietnamensis (PV) --
P. vietnamensis var. fuscidiscus (PVF) 24
P. japonicus var. major (PJM) --
P. pseudo-ginseng subsp. himalaicus (PPH) 24
Acanthopanax senticosus
1
63
1
2
2
87
4
98
1
17

Fig. 2. The strict consensus tree reconstructed on the basis of maximum parsimonious analysis of *mat*K gene sequence from seven *Panax* taxa. Tree length = 28, CI = 1.000, RI = 1.000, RC = 1.000. Number above line is branch length, and number below line is the bootstrap value with 1000 replicates. The chromosome numbers are shown in the right column (*obtained from Yang 1981, **obtained from Hara 1970).

quence was identical to that of PV and PQ, the *mat*K gene sequence of PVF exhibited specificity and intra-taxon stability. Two sites of nonsynonymous nucleotide difference were found between PVF and PV, while seven nucleotide substitutions accompanying 2 sites of amino acid variation were found between PVF and PJJ.

It was noteworthy that PVF had a somatic chromosome number of 2n = 24, which differed from 2n = 48 of *P. japonicus* (Hara 1970). PPH collected from Arunachar Pradesh, India was also of 2n = 24 (personal communication with Dr. Akihito Takano, Medicinal Plant Garden, Showa College of Pharmaceutical Sciences). These cytological evidences were congruent with the phylogenetic tree (Fig. 2) reconstructed by *mat*K gene sequences. PG, PJJ and PQ of which the chromosome numbers were 2n = 48(44) (Yang 1981) comprised one cluster, while PVF and PPH of which chromosome numbers were 2n = 24 belonged to another cluster. In general, flora at higher latitude, higher altitude and harsher environments exhibit a higher proportion of polyploids (Yang 2001). In our present study PVF, from southern Asia, was found to be a diploid species, contrasting with PG, PJJ and PQ–tetraploids species, growing in the northern parts of Asia and America. In the phylogenetic tree (Fig. 2), PJJ and PJM were divided into different clusters, while PJM showed a close relation with PPH. The taxonomic arrangement of four varieties of *P. japonicus* advocated by Wu et al., including var. *japonicus*, var. *major*, var. *angustifolius* and var. *bipinnatifidus*, has long been debated. Hara (1970) treated them as subspecies or varieties under *P. pseudo-ginseng*, and Hoo and Tseng (1973, 1978) treated them as varieties under *P. pseudo-ginseng*. To get more evidences for assisting taxonomic assignment of these plants, explorations of the relations among extent *Panax* taxa, including *P. pseudo-ginseng, P. japonicus* var. *angustifolius*, var. *bipinnatifidus*, etc., using molecular data, are in progress.

In addition, our chemical study on the same crude drug sample (TMPW No. 19759) derived from PVF showed that among 30 dammarane saponins isolated, the ocotillol-type saponins occupied a remarkably high ratio, namely, majonoside-R_2 and vina-ginsenoside R_2 accounted for more than 50 % of its total saponins. The oleanolic acid saponin was not isolated from PVF (Zou et al. 2002a, 2002b). The spectrum of constituents in PVF was markedly different from PJJ, which contained a large amount of oleanolic acid saponins with only a small amount of dammarane saponins. Ocotillol-type saponins have not been isolated from PJJ update (Morita et al. 1985). This chemical spectrum was considerably similar to that of PV which also contains a number of dammarane saponins with an ocotillol side chain. However, oleanolic saponins have been isolated from PV (Duc et al. 1993, 1999).

From the above morphological, molecular and chemical evidences, *P. vietnamensis* var. *fuscidiscus* appears to be a distinct *Panax* taxon, and shows a close relationship with PV.

Panax vietnamensis Ha & Grushv.

var. **fuscidiscus** K.Komatsu, S.Zhu & S.Q.Cai, var. nov. [Figs. 3–5]

Haec varietas nova *Panax vietnamensi* valde affinis est; a quo disco plano, fusco vel vaccino et stylis duobus completus separatis facile distinguitur.

Perennial plant, 50–80 cm high. Rhizome bamboo-like, creeping horizontally, thick, 1–2 cm in diameter; nodes closely-compacted with conspicuous stem scar, internode shorter than 6 mm, outer surface yellowish-brown or brown, fractured surface gray with somewhat pink tinges, after drying reddish-brown. Fleshy root attached below rhizome, lumpy, spindle shaped or subconic, 2–4 cm

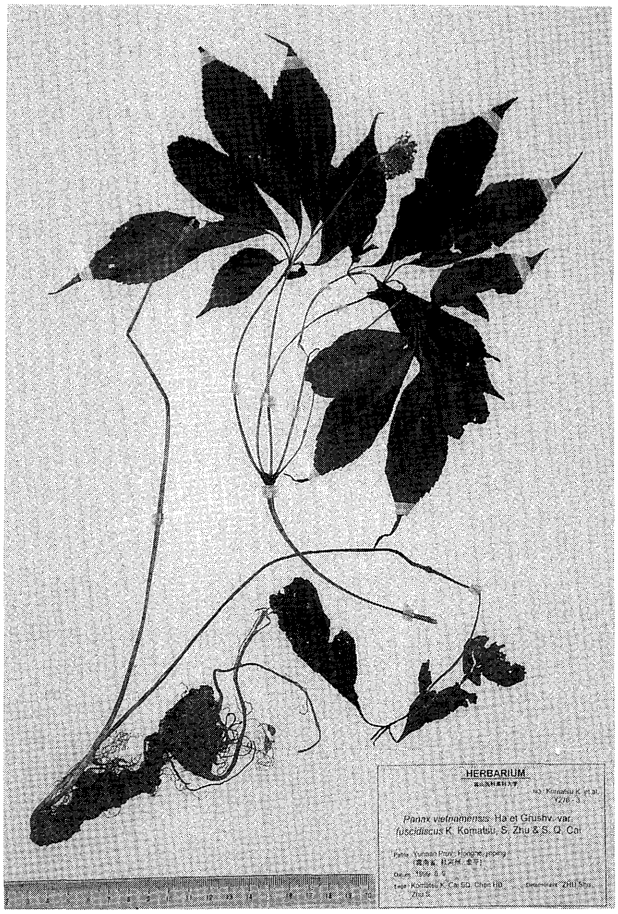
HERBARIUM
Panax vietnamensis Ha et Grushv. var.
fuscidiscus K. Komatsu, S. Zhu & S. Q. Cai

Fig. 3. Holotype of *Panax vietnamensis* Ha & Grushv. var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai.

in diameter, 3–12 cm long, heavy and compact in texture. Stem erect, slender, generally solitary, dark-green with shallow longitudinal striations, glabrous. 2–5 palmate compound leaves verticillate at the top of stem, petiole slender, 4–10 cm long, glabrous. Leaflets generally 5, rarely 7, the central three almost same in size, ovate-oblong or elliptic, 8–14 cm long in total, 3–5.5 cm wide, apex long caudate-acuminate, 2–2.5 cm in length. Outermost one pair of leaflets much smaller, ovate, 3.5–9 cm long, 2–4.5 cm wide, cuspidate at apex, slightly oblique at base. Margin of leaflets incise-serrate or doubly serrate, conspicuous bristles present along the veins of both surfaces, 1–2.5 mm and 0.5–1 mm long on upper and lower surfaces, respectively. Petiolule 0.3–2 cm long. Peduncle slender, singularly terminal on stem, about two times longer than petiole, glabrous. Umbelliform inflorescence consisting of 70–100 flowers. Flowers tiny, ca. 1–2 mm in diameter, greenish-white; pedicels 1 cm long, outer surface with small papillae. Receptacle cup-shaped, disk flat, fuscous or vaccinous. Sepals 5, blunt-angular, glabrous; petals 5, long-ovate, 2.5 mm long, 1 mm wide, greenish-white; stamens 5, filament

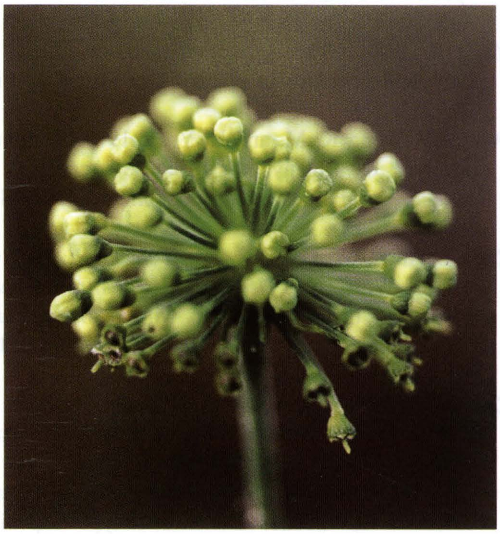

Fig. 4. Umbelliform inflorescence of *Panax vietnamensis* Ha & Grushv. var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai. The disk of flower is fuscous or vaccinous.

usually as long as or exceeding petal; ovary inferior, styles 1 or 2, completely separated in the case of 2-styled flowers, loculus 1 or 2.

Typus: China. Yunnan province, Honghe Hani-zu Yi-zu autonomous division, Jinping county. Alt. 1800 m. [Aug. 9, 1999. K. Komatsu, S. Q. Cai, H. B. Chen, S. Zhu, Y276. Holotype (Y276–3) and one isotype (Y276–1) in TMPW, and two isotypes (Y276–2, 4) in PEM].

Other specimens examined: **China**. Yunnan province, Honghe Hani-zu Yi-zu autonomous division, Lüchun county. Alt. 2100–2400 m. Oct. 17, 1973. Tao Deding 905, 942.

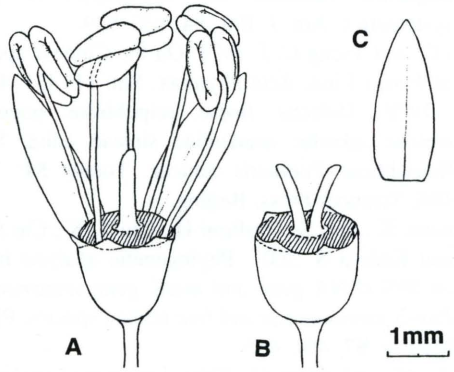

Fig. 5. Flower of *Panax vietnamensis* Ha & Grushv. var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Cai. A: 1-styled flower (partial); B: 2-styled flower (partial); C: petal.

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朱　姝[a,b]，伏見裕利[a]，蔡　少青[b]，陳　虎彪[b]，小松かつ子[a*]：中国雲南省南部のトチバニンジン属1新変種とその18S rRNA遺伝子及び*mat*K遺伝子の塩基配列

富山医科薬科大学和漢薬研究所と中国北京大学薬学院天然薬物学系との共同研究で1999年に中国雲南省においてトチバニンジン属植物の学術調査を行った．雲南省南部の金平で入手した根茎が竹節状を呈するトチバニンジン属植物の一種は，根茎の形態並びに同属植物の分布から *P. japonicus* (PJJ) と考えられた．しかし，遺伝子解析の結果，核DNAの18S rRNA遺伝子及び葉緑体DNAの*mat*K遺伝子の塩基配列はPJJと明らかに異なり，*P. vietnamensis* (PV) に近かった．これまでの研究により，両遺伝子の配列はトチバニンジン属植物では種のレベルで安定していることが証明されている．そこで，入手した植物数個体についてさらに外部形態を検討した．その結果，小葉は長卵形で，先端が長さ2–2.5 cmで尾状に尖り，上下面の脈に沿って顕著な剛毛があること，単一の散形花序に70–100個の小花がつき，花柱は1または2個，子房が1室または2室であることなどの特徴が見られ，PJJとは異なっていた．一方，これらの特徴はPVに類似したが，小花の花盤が平坦で褐色または暗褐色を呈し，花柱が2個ある場合にこれらが基部から完全に分離する点でPVと異なっていた．褐色または暗褐色を呈する花盤はこれまでに報告されているトチバニンジン属各種に見られない特徴である．以上の特徴により，雲南省南部で得られた植物を *P. vietnamensis* Ha & Grushv. var. *fuscidiscus* K.Komatsu, S.Zhu & S.Q.Caiと命名，記載した．さらに，この新変種の染色体数は2n = 24であることを確認した．

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